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(54) Title: PRIMATE CLUSTER-FORMING EMBRYONIC HEMATOPOIETIC STEM CELLS

(57) Abstract

The invention provides novel cluster-forming stem cells and stem cell growth factor isolated therefrom.

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PRIMATE CLUSTER-FORMING EMBRYONIC HEMATOPOIETIC STEM CELLS

INTRODUCTION

In mammals, there is a constant turnover of blood cells. To provide a source for these cells, it is believed that there is a single cell type, the hematopoietic stem cell, which is capable of giving rise to all the blood cell lineages. The stem cell divides to give rise to cells which are committed to a specific lineage, or to produce more stem cells by self-regeneration. The stem cell population constitutes only a small percentage of the total number of hematopoietic cells. Fetal and adult hematopoietic stem cells have been characterized by the absence or presence of markers on the cell surface. The phenotype for a highly enriched human stem cell fraction is reported as CD34+, Thy-1+ and lin-.

The blood cell lineages include lymphoid, myeloid and erythroid cells. Cells of the lymphoid lineage, B cells and T cells, produce antibodies, regulate the cellular immune system and detect foreign antigens and cells. The myeloid lineage, which includes monocytes, granulocytes, megakaryocytes as well as other cells, monitors for the presence of foreign bodies in the blood stream, provides protection against neoplastic cells, scavenges foreign materials from the blood stream and produces platelets. The erythroid lineage provides red blood cells, which act as oxygen carriers.

There are a number of clinical uses for a purified stem cell population. Gene therapy may rely on transformation of a self-renewing population such as the stem cell. Bone marrow transplantation is currently used in conjunction with chemotherapy and radiation for the

treatment of leukemia and other cancer patients. The isolation of factors and receptors required for the maintenance of stem cell properties is also of interest.

Cells of interest include very early hematopoietic cell populations, which may be isolated from embryonic tissues.

Relevant Literature

U.S. Patent no. 5,061,620 describes the characterization of human stem cells. The phenotype of stem cells with rhodamine staining is discussed in Spangrude and Johnson (1990) P.N.A.S. 87:7433-7437.

Summary of the Invention

This invention relates to a novel population of cluster-forming embryonic hematopoietic stem cells. The use of such cells in medical and research applications, and novel stem cell growth factor(s) obtained or obtainable from such cell populations is also described.

Description of the Specific Embodiments

A dense population of cells, marked by the surface expression of the CD34 glycoprotein and associated with the ventral wall of the dorsal aorta in early human embryos, is provided. This novel population of human intraembryonic hematopoietic cells appears to develop independently of the yolk sac. It is a very early stage population of stem cells and indeed appears to be the real stem of the whole blood system. It is, moreover, the first example of a population of cluster-forming stem cells.

These cluster-forming stem cells are isolated from embryos in the early stages of gestation, *e.g.* from about 10-60 days, preferably from about 4-6 weeks, most preferably about five weeks from conception. The cells of the invention are associated with and may be isolated from

endothelial tissue obtained from such embryos, *e.g.* from developing intraembryonic blood vessels, most preferably from the ventral aspect of the dorsal aorta in the pre-umbilical region.

These stem cells are capable of self-regeneration and, like known fetal and adult stem cells, will in the natural state or in the presence of the requisite cytokines become lineage-committed progenitors that are dedicated to differentiation and expansion into a specific lineage. Primate, *e.g.* human, stem cells identified to date have been found *in vivo* only in highly dispersed form. The cells of the invention, however, exist *in vivo* as clusters of undifferentiated stem cells. The invention thus provides for the first time an isolated population of primate, *e.g.* human, cluster-forming stem cells. The cells are substantially free of other cell types, usually at at least about 90% free, preferably at least about 95% free, more preferably at least about 99% free.

The CD34 glycoprotein is a convenient indicator of hematogenous cells in both the developing and the adult human organism. $CD34^+$ cells are found in fetal liver, umbilical cord or adult mobilized peripheral blood and in both fetal and adult bone marrow. $CD34^+$ cells also include the earliest multipotential hematopoietic stem cells, which do not express known differentiation-associated molecules but display the Thy-1 antigen at their surface. CD34 is also expressed in the adult by most vascular endothelial cells.

The data presented herein demonstrate that CD34 expression accompanies the early ontogeny of the human vascular system, since it was detected on endothelial cells in the yolk sac and embryo at 23 days of gestation, the earliest stage tested. By 35 days of gestation, CD34 is uniformly expressed at the luminal aspect of endothelial cells in developing intraembryonic blood vessels.

Strikingly, a CD34-labeled thickening of the ventral aspect of the dorsal aorta is also observed in the pre-umbilical region of the embryo at that stage and seen at a higher magnification to be composed of packed, CD34-positive, round cells in close apposition to the endothelium. Intraaortic CD34-positive cell clusters were observed in six different human embryos ranging from 30 to 37 days of gestation. In each case, these clusters were localized in the pre-umbilical region, just underneath the anterior limb rudiment. More rostral or more caudal sections of the dorsal aorta did not contain them.

The cells of the invention can be differentiated from cells of the aortic endothelium. The lectin from the gorse plant, *Ulex europaeus*, which specifically binds to murine and human adult vascular endothelial cells, marks most of the cells of the endothelial lining of the 35-day human dorsal aorta, but does not show affinity for the above-described intraaortic cell clusters. A reverse pattern is observed following immunostaining of the same region for the pan-leukocyte CD45 marker, which was expressed by the endothelium-associated cells, but not by the endothelium itself. Finally, both the aortic endothelium and the associated hematopoietic CD34+ cells are seen to express CD31, the platelet-endothelial cell adhesion molecule-1 (PECAM-1) which is displayed at the surface of both vascular endothelial cells and early hematopoietic precursor cells.

The cells of the invention are characterized as round adherent cells locally accumulated in the pre-umbilical region of the primate, *e.g.* human, dorsal aorta during the early stages of gestation, usually between about 10 to 60 days, more usually between about 4 to 6 weeks, preferably about 5 weeks post-fertilization for human cells. The cells are further characterized as being positive for CD34, characteristic of hematogenous cells

(CD34$^+$); and negative for the receptor recognized by *Ulex europaeus* lectin, characteristic of endothelial cells (Ulex$^-$).

The cells of the invention are optionally further characterized as lacking at least one or more of characteristic lineage markers (Lin) for committed cells, e.g., CD15 or CD33 for myeloid pathways, CD10 or CD19 for B-cell lineage, or glycophorin A or Ulex lectin for erythroid lineage. A cell which is identified as lacking one or more of these lineage markers is referred to herein as being Lin$^-$. Alternatively, or in addition, the cells are identified as lacking CD38, a global indicator of committed progenitors (CD38$^-$). The cells of the invention can optionally be selected for by the expression of the adhesion molecules CD43, CD44 and ICAM-1. The cells of the invention can also optionally be identified by their low affinity for the supravital dye rhodamine 123 (Rho^{lo}).

The cells of the invention are thus suitably isolated and characterized as being CD34$^+$, CD31$^+$, CD45$^-$ and Ulex$^+$. They may be further characterized as Lin$^-$ and/or CD38$^-$ and/or Rho^{lo}.

The cells of the invention can also be segregated from the associated endothelial cells by the use of probes for the *c-myb* (hematopoietic-specific) and *ets-1* (endothelium-specific) oncogenes, used for *in situ* hybridization.

In a further embodiment, the invention comprises the progeny of the cells of the invention, when the progeny is generated in *ex vivo* cell culture.

Serial, CD34-immunostained, transverse embryo sections were used to computerize a spatial image of the pre-umbilical region of a 35-day human dorsal aorta. CD 34-positive, non-endothelial intraaortic cells appear to be densely gathered on the floor of the blood vessel, and virtually absent from its dorsal aspect. Their number has

been estimated, from computer data, at 831 cells on a distance spanning one to two somites in the immediate pre-umbilical area.

It is believed that the cells of the invention are forerunners of the fetal and post-natal human blood system. Rare scattered non-endothelial CD45+ CD34+ cells from the fifth week of gestation in the liver rudiment were also detected, indicating simultaneous hepatic hematopoietic development. Based on CD 34 detection in embryonic, fetal and adult human tissues, the intraaortic cell clusters described herein represent the densest local accumulation of hematopoietic CD34+ cells encountered throughout the development of the human blood system. Even at the crucial phases of hematopoietic development in the yolk sac, embryonic liver and fetal bone marrow, CD34+ hematopoietic cells remain extremely rare and scattered in those blood-forming tissues. Thus discovery of this population of cells represents the first opportunity to obtain isolated populations of these embryonic stem cells.

Various techniques are employed to separate the cells from a sample of tissue from the dorsal aorta region of an embryo. The cells are visually identifiable as the cluster of round adherent cells on the dorsal aortic wall and a crude separation can be made by pipetting or otherwise dissecting this cluster from the endothelial tissue.

If desired, the cells are further purified by affinity separation techniques. Monoclonal antibodies are particularly useful for identifying markers associated with particular cell lineages and/or stages of differentiation. The antibodies may be attached to solid support to allow for crude separation. The separation techniques employed should maximize the viability of the fraction to be collected. Various techniques of different efficacy may be employed to obtain "relatively crude" separations. Such

separations are where up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells present not having the marker may remain with the cell population to be retained. The particular technique employed will depend upon efficiency of separation, cytotoxicity of the methodology, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

Procedures for separation may include, but are not limited to, magnetic separation, using antibody-coated magnetic beads, flow cytometry, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, *e.g.* plate, elutriation or any other convenient technique.

The use of separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (*e.g.* lectin and antibody affinity), and vital staining properties (*e.g.* mitochondria-binding dye rhodamine 123 and DNA-binding dye Hoechst 33342).

Techniques providing accurate separation include, but are not limited to, FACS, which can have varying degrees of sophistication, *e.g.* a plurality of color channels, low angle and obtuse light scattering detecting channels, low angle and obtuse light scattering detecting channels, impedance channels, *etc.*

One procedure that may be used is first incubating the cells for a short period of time at reduced temperatures, generally about 4°C, with saturating levels of antibodies specific for a particular committed cell type, including, but not limited to, CD3 and CD8 for T cell determinants, and then washing the cells with a FCS

cushion. The cells may then be suspended in a buffer medium and separated on the basis of the antibodies for the particular determinants, using various protein(s) specific for the antibodies or antibody-antigen complex.

Conveniently, the antibodies are conjugated with markers, including, but not limited to, magnetic beads, which allow for direct separation, biotin, which can be removed with avidin or streptavidin bound to a support, fluorochromes, which can be used with a FACS, or the like, to allow for ease of separation of the particular cell type. Any technique may be employed which is not unduly detrimental to the viability of the remaining cells.

In a first separation, the antibody for CD34 may be labeled with one fluorochrome, while the antibodies for the various dedicated lineages may be conjugated to a different fluorochrome. Fluorochromes which find use in a multi-color analysis include, but are not limited to, phycobiliproteins, *e.g.* phycoerythrin and allophycocyanins; fluorescein, Texas red, *etc.* The separation can be performed in any order, but generally, the sequence of the procedure is a negative selection step to remove undesired, *e.g.* endothelial cells and lineage-committed cells, in combination with positive selection for CD34 and optionally CD31 and/or CD45.

The cells may be selected against dead cells, by employing dyes associated with dead cells, including but not limited to, propidium iodide (PI). Preferably, the cells are collected in a medium comprising 2% FCS.

Other techniques for positive selection may be employed, which permit accurate separation, such as affinity columns, and the like. The method should permit the separation from a residual amount of the non-stem cell populations. Cells may be selected based on light-scatter properties as well as their expression of various cell surface antigens.

The invention provides a method for isolation or identification of the cells of the invention, comprising subjecting a population of cells obtained or derived from cells locally accumulated in the pre-umbilical region of the dorsal aorta of a human embryo during the early stages of gestation, to the following steps, in any order: (i) a negative selection step whereby the population of cells is contacted with one or more antibodies, *e.g.* antibody to a Lin marker or CD38, and/or lectin(s), *e.g.* Ulex lectin, that recognize undesired cells but not the cells of the invention, especially CD38 and/or Ulex lectin, and (ii) a positive selection step whereby the population of cells is contacted with one or more antibodies capable of recognizing the cells of the invention, *e.g.* antibody to CD34, CD45, CD43, CD44, ICAM-1, and/or CD31, especially CD34. The method may utilize flow cytometry techniques or any of the selection techniques described above wherein the antibodies are conjugated to a label, bead, or solid support.

The subject methods include the use of an antibody recognizing the cells of the invention, *e.g.* CD34, CD31, and/or CD45 antibody, and/or of antibody or lectin recognizing undesired cells but not the cells of the invention, *e.g.* antibody to a Lin marker or CD38 antibody or Ulex lectin, in a method for identifying or isolating the cells of the invention as described herein, or in a kit or device for isolating the cells of the invention.

For convenience, a kit comprising reagents, parts or devices for isolation or identification of the cells of the invention may be assembled. The kit may comprise at least one positive selection antibody capable of recognizing the cells of the invention as described herein; at least one negative selection antibody which recognizes undesired cells but not the cells of the invention as described

herein, and optionally, instructions for use in a method as described above.

Once the cells of the invention have been isolated, they are propagated in suitable growth media. Media for the culture of hematopoietic cells *in vitro* are known in the art, including IDDM, Iscove's medium, DMEM, RPMI, *etc.* Culture medium will contain serum, *e.g.* FCS, bovine serum, autologous serum, *etc.*, or a suitable serum-free replacement. See, for example, Ponting *et al.* (1991) Growth Factors **4**:165-173. Media may also contain such additives as b-mercaptoethanol, antibiotics, vitamins, and growth factors that support the maintenance of stem cells. Growth factors may be supplied as defined factors, *e.g.* IL-1, IL-3, IL-6, G-CSF, GM-CSF, c-kit ligand, LIF, etc., generally at final concentrations of from about 1 ng per ml to as high as about 1 mg/ml. Growth factors may be added alone, or in combinations, depending on the desired effect.

Alternatively, growth factors may be supplied by stromal or endothelial cells that secrete the necessary growth factors. Medium may be conditioned medium from stromal cells, such as stromal cells that can be obtained from bone marrow, fetal thymus or fetal liver, and are known to provide for the secretion of growth factors associated with stem cell maintenance. The stem cells may be co-cultured with such cells, or in medium comprising maintenance factors supporting the proliferation of stem cells. The stromal cells may be allogeneic or xenogeneic. Before using in the co-culture, the mixed stromal cell preparations may be freed of hematopoietic cells employing appropriate monoclonal antibodies for removal of the undesired cells, *e.g.* with antibody-toxin conjugates, antibody and complement, *etc.* Alternatively, cloned stromal cell lines may be used where the stromal lines are be autologous, allogeneic or xenogeneic.

The cells of the invention find use as therapeutic agents by transplantation to regenerate the hematopoietic system of a host deficient in stem cells. Conditions where such therapy is used include rescuing a subject that is diseased, *e.g.* suffering from lymphoma, leukemia, or other neoplastic condition, and can be treated by removal or destruction of bone marrow and hematopoietic tissue by irradiation or chemotherapy, followed by engraftment with the cells of the invention. The symptoms of immunodeficiency disorders or diseases, *e.g.* combined immunodeficiency (CID), acquired immunodeficiency syndrome (AIDS), and congenital immunodeficiencies are treated or alleviated by engraftment of the cells of the invention in a subject suffering from such diseases or disorders. Damage to the hematopoietic system, *e.g.* as a result of radiation, chemotherapy, immunosuppressive drugs, surgery or trauma, is repaired or alleviated by transplation of the subject cells.

The cells of the invention may be used for the treatment of genetic diseases. Genetic diseases associated with hematopoietic cells may be treated by genetic modification of autologous or allogeneic stem cells to correct the genetic defect. For example, diseases including, but not limited to, β-thalassemia, sickle cell anemia, adenosine deaminase deficiency, recombinase deficiency, recombinase regulatory gene deficiency, *etc.* may be corrected by introduction of a wild-type gene into the cells of the invention, either by homologous or random recombination. Other indications of gene therapy are introduction of drug resistance genes to enable the cells of the invention to have an advantage and be subject to selective pressure during chemotherapy. Suitable drug resistance genes include, but are not limited to, the gene encoding the multidrug resistance (MDR) protein. Diseases other than those associated with hematopoietic cells may

also be treated, where the disease is related to the lack of a particular secreted product including, but not limited to, hormones, enzymes, interferon, growth factors, or the like. By employing an appropriate regulatory initiation region, inducible production of the deficient protein may be achieved, so that production of the protein will parallel natural production, even though production will be in a different cell type from the cell type that normally produces such protein. It is also possible to insert a ribozyme, antisense or other message to inhibit particular gene products or susceptibility to diseases, particularly hematolymphotropic diseases.

Alternatively, one may wish to remove a particular variable region of a T-cell receptor from the T-cell repertoire. By employing homologous recombination, or antisense or ribozyme sequence which prevents expression, the expression of the particular T-cell receptor may be inhibited. For hematotropic pathogens, such as HIV, HTLV-I and II, *etc.* the stem cells could be genetically modified to introduce an antisense sequence or ribozyme which would prevent the proliferation of the pathogen in the stem cell or cells differentiated from the stem cells. Methods for recombination in mammalian cells may be found in Molecular Cloning, A Laboratory Manual (1989) Sambrook, Fritsch and Maniatis, Cold Spring Harbor, NY.

The cells of the invention are naive and undifferentiated. As they have not yet become specific for a particular individual, they will not mature into white blood cells which attack the cells of the recipient, as may occur in graft vs. host disease following bone marrow transplant. Maturation, proliferation and differentiation of the cells of the invention into one or more selected lineages is preferably accomplished by employing a variety of hematopoietic factors and cytokines, including, but not limited to erythropoietin, leukemia inhibitory factor

(LIF), colony stimulating factors, *e.g.* GM-CSF, G-CSF or M-CSF, interleukins, e.g., IL-1, -2, -3, -4, -5, -6, -7, -8, etc., or the like, Steel factor (MGF or c-kit ligand), or stromal cells associated with the stem cells becoming committed to a particular lineage, or with their proliferation, maturation and differentiation.

The cells may be frozen at liquid nitrogen temperatures and stored for long periods of time, being thawed and capable of being reused. The cells will usually be stored in 5% DMSO and 95% fetal calf serum. Once thawed, the cells may be expanded by use of growth factors or stromal cells associated with stem cell proliferation and differentiation. The cells may optionally be modified to have at least one non-autologous gene, as described above for use in gene therapy, and such genetically altered populations and their progeny are embraced within the scope of this invention.

Pharmaceutical preparations are also provided, comprising the cells of the invention in a form suitable for administration, *e.g.* by injection or infusion, to a patient in need thereof, in combination with a suitable carrier medium for use in any of the foregoing treatments. Also provided are the cells of the invention for pharmaceutical use, and use of the cells of the invention in the manufacture of a pharmaceutical preparation, e.g., for use in any of the foregoing treatments.

Dosages of the cells of the invention for pharmaceutical uses such as reconstitution of a the hematopoietic system of a patient in need thereof, will vary depending on the nature of the condition to be treated and the other aspects of the patient's treatment, *e.g.* prior radiation or chemotherapy, or co-therapy with agents having an influence on hematopoiesis, *e.g.* cytokines, as well as on the purity and viability of the cell population to be administered. Because, in principle, a single cell

of the invention is capable of regenerating the entire hematopoietic system of a patent, the precise dosage of cells to be administered is not critical. Preferably, however, at least 10^3 cells, more preferably at least 10^5 cells are administered by infusion, and the patient preferably also receives cytokines, e.g., GM-CSF and IL-3. Subsequent infusions may be performed as required.

The cells of the invention are also of use as research tools in producing various hematopoietic cell lines; detecting and evaluating growth factors relevant to stem cell self-regeneration; developing hematopoietic cell lines and assaying for factors associated with hematopoietic development; providing animal models engrafted with the cells of the invention, *e.g.* SCID mice or other immunocompromised animals which are engrafted with cells of the invention, thereby developing a model human hematopoietic system thatcan be usefully employed to study the human hematopoietic and immune systems and diseases thereof, and to test drugs which modulate or affect such systems and treat or alleviate such diseases; identifying support cells (endothelial, stromal, fibroblast) in the developing embryo that allow for this localized concentration of CD34$^+$ cells, which support cells can then be examined for novel cytokine production or novel adhesion molecules important for regulating the growth of these cells.

The cells may also be used in the isolation and evaluation of factors associated with the differentiation and maturation of hematopoietic cells. Thus, the cells may be used in assays to determine the activity of media, such as conditioned media, evaluate fluids for growth factor activity, involvement with dedication of lineages, or the like. The subject cells may also be used in the identification of supportive cells for the isolation and evaluation of factors associated with the self-renewal of

hematopoietic cells. Thus, the stem cells of the invention may be used in assays to determine either autocrine or paracrine regulatory signals and evaluate responses to growth factor either from external or intrinsic protein sources; and to determine the activity of media, such as conditioned media, evaluate fluids for cell growth activity, involvement with dedication of particular lineages, or the like.

The subject cells produce and/or are responsive to a factor that permits replication of stem cells without differentiation. This factor may be isolated from media or cell extracts of supportive cells in which a population of the cells of the invention are growing or a supernate of a population of the cells, by separating or fractionating the fluid, e.g. chromatographically. The active fraction containing the desired factor is identified by measuring the growth and differentiation of stem cells in the presence and absence of such fractions, or alternatively, using comparative analysis of fluid obtained from a population of adult stem cells. Additionally, cDNA libraries of the cells of the invention may be prepared and compared to cDNA libraries from stem cells isolated from adults, and the gene for the factor (or its receptor) identified thereby. Growth factor or receptor genes in the cDNA libraries may optionally be amplified and identified using oligonucleotide primers based on conserved sequences within known growth factor or receptor families.

To produce cDNA libraries, RNA is isolated from the subject cells. Residual DNA may be removed in accordance with conventional techniques and the polyadenylated RNA purified further, on oligo-dT sepharose, gel chromatography, *etc.* cDNA may then be prepared in accordance with conventional techniques using reverse transcriptase (see Sambrook, *et al., supra*). The polymerase chain reaction may be used to amplify the amount

of cDNA that is produced. The cDNA is then introduced into an appropriate cloning system. The cDNA may be used for further probing of the cDNA library for a complete transcript. Alternatively, the cDNA sequence may be used to probe a genomic library to identify the genomic gene encoding the subject proteins (See, for example, Sambrook *et al. supra.*)

cDNA libraries will generally include complete or partial copies of at least about 10^2 different DNA species, more usually at least about 10^3 different species, and may comprise as many as 10^4. Each cDNA may be represented from 1 to 10^3 times in the initial library.

The nucleic acid compositions of the subject invention may be genomic or cDNA sequences encoding all or a part of the subject adhesion and homing molecules. Fragments may be obtained of the cDNA or genomic sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction enzyme digestion, by PCR amplification, *etc.* For the most part, fragments will be of at least 12 nt, more usually at least 18 nt. Preferably fragments will include a functional epitope.

The DNA may also be used to identify cells or organs which are expressing the subject genes. The manner in which one probes cells for the presence of particular nucleotide sequences, particularly as DNA, mRNA or cDNA, is well-established in the literature and does not require elaboration here. Conveniently, mRNA may be isolated free of DNA, and by using reverse transcriptase and PCR with primers specific for the various allergens, the subject cDNAs of may be expanded, separated on gel electrophoresis and then probed using Southern blotting or sequencing. Other techniques may also find use.

For expression, the DNA sequences may be inserted into an appropriate expression vector, where the native transcriptional initiation region may be employed or an

exogenous transcriptional initiation region, *i.e.* a promoter other than the promoter which is associated with the gene in the normally occurring chromosome. The promoter may be introduced by recombinant methods *in vitro*, or as the result of homologous integration of the sequence into a chromosome. A wide variety of transcriptional initiation regions are known for a wide variety of expression hosts, where the expression hosts may involve prokaryotes or eukaryotes, particularly *E. coli*, *B. subtilis*, mammalian cells, such as CHO cells, COS cells, monkey kidney cells, lymphoid cells, particularly human cell lines, and the like. Generally a selectable marker operative in the expression host will be present. The promoter may be operably linked to the coding sequence of the genes of interest so as to produce a translatable mRNA transcript. Expression vectors have convenient restriction sites located near the promoter sequence so as to provide for the insertion of nucleic acid sequences encoding heterologous proteins. The promoters in suitable expression vectors may be either constitutive or inducible.

The cDNA clones may be introduced into a variety of vectors, where the vectors will normally be characterized by the ability to provide selection of cells comprising the expression vectors. The vectors may provide for extrachromosomal maintenance, particularly as plasmids in bacteria or viruses in eukaryotic cells, or for integration, particularly in mammalian cells. Where extrachromosomal maintenance is desired, an origin sequence will be provided for the replication of the plasmid, which may be a low- or high-copy plasmid. A wide variety of markers are available for selection, particularly those which protect against toxins, more particularly against antibiotics. The particular marker which is chosen will be selected in accordance with the nature of the host, where in some cases, complementation may be employed with

auxotrophic hosts, *e.g.* yeast. Introduction of the DNA construct may be by any convenient means, *e.g.* calcium-precipitated DNA, electroporation, fusion, transfection, infection with viral vectors, *etc.*

In another embodiment, polyclonal and/or monoclonal antibodies capable of specifically binding to a protein(s) or fragments thereof are provided wherein the cells of the invention are used as antigen to provide an antibody recognizing an epitope characteristic of the cells of the invention. The term antibody is used to refer both to a homogeneous molecular entity, or a mixture such as a serum product made up of a plurality of different molecular entities. Monoclonal or polyclonal antibodies specifically reacting with the protein(s) may be made by methods known in the art, see for example, Antibodies: A Laboratory Manual, CSH Laboratories; Monoclonal Antibodies: Principles and Practice, 2d ed, Academic Press, New York. Also, recombinant immunoglobulins may be produced by methods known in the art, including, but not limited to, the methods described in US Patent No. 4,816,567. Monoclonal antibodies with affinities of 10^8 M^{-1} preferably 10^9 to 10^{10} or more are preferred for cell identification or negative selection; lower affinities are preferred for positive cell selection. Such antibodies using the cells of the invention are useful, *e.g.* in identifying and purifying populations of stem cells.

Thus, in a yet further embodiment, the invention provides for a stem cell growth factor, characterized as being non-differentiating, i.e., facilitating and/or promoting growth of the stem cell population without differentiation. This factor is further characterized in that it is capable of being isolated from growth media or cell extract in which a population of cells of the invention is growing, or from supernate from a culture of the cells of the invention. The factor thus isolated is

preferably in pure or substantially pure form, *e.g.* at least 90%, preferably at least 95%, most preferably at least 99% pure form.

Immortalized cells of the invention are further useful as being cells which are responsive to a factor allowing for the regeneration of stem cells, *e.g.* in assays for survival, activation, or proliferation in the presence and absence of the putative stem cell growth factor.

EXPERIMENTAL

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to insure accuracy with respect to the numbers used (*e.g.* amounts, temperature, concentrations, *etc.*) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.

Example 1: Identification of CD34' cell clusters in five week human embryo

Human embryos of 23 to 50 days gestation were obtained immediately after voluntary terminations of pregnancy induced by administration of the antiprogesterone compound RU 486. Gestational age was estimated from developmental anatomic criteria. In all cases, informed consent to the use of the embryo in research was obtained from the patient, and embryos were collected according to the guidelines, and with the authorization, of the French Comité National D'Ethique. Gestational (i.e. post-conception) age is estimated from

menstrual history and confirmed on development anatomic criteria. Embryos fixed overnight at 4°C in phosphate-buffered saline (PBS) 4% paraformaldehyde (v/v) are rinsed in PBS, dehydrated and embedded in paraffin. 5 μm-thick sections are deparaffinized and endogenous peroxidases are inhibited for 20 min. in methanol containing 0.2% hydrogen peroxide. Sections are then washed with PBS 0.25% Triton X-100 and non-specific staining is blocked with non-immune goat serum. The anti-CD34 antibody (HPCA-1, Becton-Dickinson) is added overnight at 4°C. After washing with PBS-Triton X-100, incubation is carried out for 1 hour at room temperature with, first, biotinylated rabbit anti-mouse Ig antibody (DAKO) and subsequently with peroxidase labeled streptavidin (DAKO). Peroxidase activity is revealed with 0.025% (v/v) 3,3'-diaminobenzidine (SIGMA) in PBS containing 0.015% hydrogen peroxide. Slides are counterstained with Harris hematoxylin and mounted in aqueous medium (BioGenex Laboratories) for examination on an Optiphot-2 microscope (NIKON).

Low magnification of an immunostained transverse section in the immediate pre-umbilical region reveals CD34 expression by the endothelial cells lining the dorsal aorta, the blood vessels and capillaries present around the neural tube and mesonephric rudiment. A magnified view of the aorta on the same section shows the ventral thickening of the vessel wall resulting from the accumulation of round, CD34-positive cells. Nucleated erythrocytes are present inside the lumen. In a more caudal region of the same embryo, no CD34+ cells are seen associated with the wall of the aorta. Uniform CD34+ cell clusters are most clearly evident in embryos ranging from 30-37 days gestation.

Example 2: Distinction between CD34' cell clusters and endothelial cells on the floor of the 5-week human embryonic aorta.

Endothelium-specific, biotin-labelled *Ulex europaeus* agglutinin I (commercially available from VECTOR) is incubated with transverse sections from an embryo as for the CD34 antibody preparation of example 1. The lectin is seen to bind to aortic endothelial cells, but not to the CD34-cells clumped on the vessel floor. Further immunohistology substantially as described above using antibodies to CD31 and CD45 show that CD31 is expressed on both endothelial cells and adherent intraaortic cells, but the latter also express the pan-leukocyte CD45 molecule which is absent from vascular endothelium. Monoclonal antibodies to CD45 (HLE-1) and to CD31 (JC/70A) are available commercially from Becton-Dickinson and from DAKO, respectively.

Example 3: Growth of cluster-forming stem cells in culture

The trunk area containing the segment of aorta where the CD34+ cell clusters are detected is removed from human embryos of 30-40 days gestational age. Tissues are dissociated gently by pipetting. The tissue fragments are seeded in 96-well plates precoated with a confluent layer of MS-5 murine stromal cells using the techniques described in Isaad *et al.* Blood **81**:2916 (1993). Cultures are performed in long term culture medium [12.5% FCS (Techgen, Les Ulis, France), 12.5% horse serum (Hyclone laboratories, Logan, UT), 10^{-4}M 2-β mercaptoethanol in αMEM] at 37°C. The medium is changed twice weekly. No exogenous cytokines are added. At time intervals, cell samples are harvested, pooled, counted, and processed for phenotypic analysis (in particular, CD34 and CD38 expression), and optionally further purified, by flow

cytometry using a FACSort (Becton-Dickenson) device equipped with Cellquest software. Monoclonal antibodies to CD34 [HPCA-1 and phycoerythrin (PE)- HPCA-2] and to CD38 directly coupled to fluorescein isothiocyanate (FITC), suitable for use with a FACS, are commercially available from Becton-Dickonson (San Jose, CA).

After short term co-culture, 4-10 days, the production of clonogenic progenitors was used as an indicator of the hematopoietic activity of the tissues analyzed. The results are shown in Table 1.

Table 1

	Absolute no. of Progenitor Cells Produced		
Tissue	Exp. 1	Exp. 2	Exp. 3
preumbilical aortic region	2000	460	280
liver	350	ND	44
heart	0	ND	24
limbs	5	0	ND
blood	ND	ND	4
umbilical cord	40	ND	ND

It is seen that the preumbilical aortic region generated a high number of progenitor cells giving rise to large colonies in methylcellulose assays. A clearly higher number of clonogenic progenitors was recovered from the aortic region of the oldest embryo analyzed (35-40 days). At least 30-50% of the colonies included erythroid cells. A high output of nonadherent, round, nucleated cells were observed in the wells, more than 10% of which expressed CD34, as detected by flow cytometry.

In contrast, the production of clonogenic progenitors and nonadherent CD34+ cells was strikingly lower in the wells seeded with liver cells dissected from the same embryos. Cocultures initiated with cells dissociated from the limbs or other parts of the embryo did not generate significant numbers of hematopoietic cells or progenitors in these short-term cultures. These results show that at these stages of development, the aorta-associated tissues of the trunk contain high numbers of primitive precursor cells capable of producing clonogenic progenitors of both granulocytic and arythroid lineages in a short-term stroma-dependent culture assay.

WHAT IS CLAIMED IS:

1. An isolated population of primate cluster-forming stem cells, substantially free of other cell types.

2. An isolated population of cells according to claim 1, wherein said cells are obtained from a population of stem cells locally accumulated in the pre-umbilical region of the dorsal aorta of a human embryo during the early stages of gestation.

3. An isolated population of cells according to either of claims 1 or 2, wherein said cells are further characterized as positive for CD34, CD45, and CD31; and negative for the marker recognized by *Ulex europaeus* lectin.

4. A co-culture of cells consisting essentially of a population of cells according to any of claims 1-3 and a population of stromal cells.

5. A pharmaceutical preparation comprising cells according to any one of claims 1 through 3.

6. Cells according to any of claims 1 through 3 for use as a pharmaceutical or therapeutic agent.

7. A method of selecting or purifying a population of cells according to any of claims 1-3, the method comprising:

subjecting a population of cells obtained or derived from cells locally accumulated in the pre-umbilical region of the dorsal aorta of a human embryo during the early stages of gestation, to the following steps, in any order:

(i) a negative selection step whereby the population of cells is contacted with one or more antibodies and/or lectin(s) that recognize undesired cells but not the cells of the invention, and

(ii) a positive selection step whereby the population of cells is contacted with one or more antibodies capable of recognizing the cells of the invention.

8. A method of treatment comprising administration of cells according to any one of claims 1 through 3 to a subject in need of any of the following treatments:

(i) regeneration of the hematopoietic system of a subject deficient in stem cells;

(ii) rescue of a subject that is diseased (e.g., suffering from lymphoma, leukemia, or other neoplastic condition) and can be treated by (a) removal of bone marrow, or destruction of bone marrow and hematopoietic tissue by irradiation or chemotherapy, followed by (b) engraftment with the cells;

(iii) treatment or alleviation of the symptoms of immunodeficiency disorders or diseases, e.g. combined immunodeficiency (CID), acquired immunodeficiency syndrome (AIDS), and congenital immunodeficiencies, by engraftment of the cells of the invention in a subject suffering from such diseases or disorders;

(iv) repair or alleviation of damage to the hematopoietic system, e.g. as a result of radiation, chemotherapy, immunosuppressive drugs, surgery or

trauma, in a subject in need of such repair;

(v) gene therapy, e.g., by engraftment in a subject following genetic transformation of the cells with the desired genes;

9. Use of cells according to any of claims 1 through 3 for

(i) producing hematopoietic cells;

(ii) detecting and evaluating growth factors relevant to stem cell self-regeneration;

(iii) developing hematopoietic cell lineages and assaying for factors associated with hematopoietic development;

(iv) providing animal models engrafted with the cells.

10. Non-differentiating stem cell growth factor in substantially pure form.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/20716

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/85; A61K 48/00
US CL : 435/420.2; 424/93.1; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/420.2; 424/93.1; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 5,061,620 (TSUKAMOTO ET AL.) 29 October 1991 (29.10.1991), see entire document, especially Abstract; column 3, lines 33-35; column 7, lines 10-11 and 21-24; column 10, line 32 --- column 3, lines 1-9	1-6, 8, 9 --- 1-9
Y	MORRISON, S. et al. The Biology of Hematopoietic Stem Cells. Annual. Rev. Cell. Dev. Biol. 1995, Vol. 11, pages 35-71, especially page 48, first full paragraph; page 44, lines 1-2; pages 45-46.	1-9
Y	ROITT, I. Essential Immunology. London: Blackwell Scientific Publications. 1994. page 163, see Table 9.1.	1-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 FEBRUARY 1997	Date of mailing of the international search report 11 MAR 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY AXELROD Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/20716

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WATT et al. The heparin binding PECAM-1 adhesion molecule is expressed by CD34$^+$ hematopoietic precursor cells with early myeloid and B-lymphoid cell phenotypes. Blood. 01 November 1993, Vol. 82, No. 9, pages 2649-2663, especially Abstract.	1-9
Y	HOLTHOFER, H. et al. *Ulex europaeus* I lectin as a marker for vascular endothelium in human tissues. Laboratory Investigation. 1982, Vol. 47, No. 1, pages 60-66, especially Abstract.	1-9
Y	GODIN, I. Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. Nature. 01 July 1993, Vol. 364, pages 67-70, especially Abstract.	1-9
Y	MEDVINSKY, A. et al. An early pre-liver intra-embryonic source of CFU-S in the developing mouse. Nature. 01 July 1993, Vol. 364, pages 64-67, especially Abstract.	1-9
Y	CHARBORD, P. et al. Early ontogeny of the human hematopoietic system. Comptes Rendus des Seances del la Societe de Biologie et du ses Filiales. 1995, Vol. 189, pages 601-609, especially Abstract and page 606, first paragraph.	1-9
Y	US, A, 5,199,942 (GILLIS) 06 April 1993 (06.04.93), see entire document, especially Abstract.	1-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/20716

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-9

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/20716

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Medline, Biosis, Scisearch, Embase, Caplus, APS
search terms: hematopoietic, stem, cluster-forming, human#, primate#, ulex?, lectin#, agglutinin, intraembryo?, dorsal ,aort##, para-aortic

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-9, drawn to primate cluster-forming hematopoietic stem cells, a method of making the cells, a pharmaceutical composition comprising the cells, and methods of treatment using the cells.

II. Claim 10, drawn to a stem cell growth factor.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical feature of the Group I invention is primate cluster-forming hematopoietic stem cells, while the special technical feature of the Group II invention is the stem cell growth factor. The compositions of the two groups are materially different. Because they do not share the same or corresponding technical feature, unity of invention is lacking.

Accordingly, the claims do not share a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.